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                                          Los Angeles, CA 90024

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Human prostate epithelial cells that can develop into "prostaspheres" display characteristics of stem/progenitor cells, including self-renewal and ability to induce prostate tubule formation in vivo. FISH analysis of prostaspheres derived from patient specimens containing the TMPRSS-ERG translocation, however, are not preserved in sphere-forming cells. In order to evaluate whether prostate cancer stem cells containing the TMPRSS-ERG translocation can be isolated, we have proposed a series of experiments to isolate tumor cells for characterization and in vivo expansion. Previously, we have found that although cancer cells can be isolated from tumor tissue, preservation of these cells in vitro and generation of xenografts is rare. In order to optimize retrieval of tumor cells and support tumor regeneration in vivo, we have implemented multiple strategies, including optimization of tissue processing to single cells, cell fractionation, and refinement of the microenvironment. This has enabled successful enrichment of TMPRSS-ERG+ tumor fractions and may lead to further identification of tumor-initiating cells present in primary prostate tumors.

15. SUBJECT TERMS
Prostate Cancer, prostate stem cells, TMPRSS-ERG, prostate tissue regeneration

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I - INTRODUCTION:

The aims of this proposal are based on the observations from our initial studies with human prostate cancer surgical specimens. We discovered that prostate stem/early progenitor cells recovered from tumor specimens lack the TMPRSS-ERG translocation found in the original tumor, when examined by fluorescence in situ hybridization (FISH). Our findings suggest either ETS rearrangements are not present at the stem/progenitor cell level, or that genetically deranged prostate stem/progenitor cells are particularly vulnerable to apoptosis or senescence in vitro, resulting in selective advantage of benign cells. We are currently evaluating the growth requirements that may enable survival and expansion of prostate stem/progenitor cells that contain ETS rearrangements. Generating an extensive collection of human prostate cancer stem cells would provide valuable biological tools for understanding the mechanisms of tumorigenesis and identifying new therapeutic targets. Factors that have affected the ability to isolate and expand primary tumors include low tumor grade/volume present in surgical samples, increased sensitivity of tumor cells to apoptosis after tissue dissociation, semi-competent immune systems in many SCID mouse strains that inhibit tumor take, and a microenvironment that lacks critical growth factors and cellular interactions.

Our laboratory has made significant progress towards tackling this problem, as evident in the published and unpublished data presented over the course of this grant. In the past year, we have made efforts to reduce warm ischemia time in order to improve sample quality. By incorporating NOD-SCID^{IL2grNULL} mice, we have greatly increased the efficiency of human cell engraftment. We have isolated primary stromal cells from 8 human fetal prostate specimens, in an effort to improve the microenvironment in support of tumorigenesis. These fetal cells support normal prostate tubule formation, induced by benign (adult) human prostate epithelial cells, and eliminate the need for murine additives (i.e., urogenital sinus mesenchyme). More importantly, early data demonstrates, for the first time, prostate <u>tumor</u> regeneration. We have obtained tumor grafts that recapitulate the patient's original cancer specimen, without any genetic manipulation of the patient's tumor cells.

II - BODY:

<u>Background and Specific Aims:</u>

Expansion of Prostate Stem/Progenitor Cells: The study of prostate stem cells (SCs) is facilitated by culturing dissociated primary cells as spheres[1, 2]. Spheres are multicellular globes that form in anchorage-independent conditions, and these cultures have been commonly used to study mammary and nervous system development[3, 4]. In our human prostate studies, spheres can be dissociated and passaged for multiple generations (self-renew), as well as be induced to form fully differentiated glands in vivo[5].

The TMPRSS-ERG Fusion is Not Identified in Prostaspheres: Since prostaspheres were generated from primary tumors, we presumed that in vitro cultures would include clonally derived benign and cancerous prostaspheres, reflective of the heterogeneity of glands found in tissue specimens. We were not able, however, to distinguish prostaspheres based on phenotype, marker expression, or growth rate. With the discovery of prevalent gene rearrangements involving ETS family members in prostate cancer, we anticipated that cytogenetic tools may enable identification of cancerous prostaspheres[6]. Gene fusions involving ERG, ETV1, and ETV4 involve a variety of 5' partners that direct aberrant expression of these transcription factors and possibly initiate a cascade of events leading to tumorigenesis [6]. The most common rearrangement involves juxtaposition of the androgen-regulated TMPRSS2 gene with ERG. TMPRSS-ERG gene fusions have been detected in primary prostate tumor specimens, metastases, and xenografts by fluorescence in situ hybridization (FISH)[6]. Analysis of prostate tumor surgical cohorts have found 36-78% of prostate cancers possess the TMPRSS-ERG fusion[6]. We wondered whether we could use TMPRSS-ERG to distinguish normal and malignant prostaspheres. The presence of this fusion in individual prostaspheres may suggest that cancer stem/early progenitor cells can be expanded in our cultures.

To test the feasibility of this approach, FISH analysis was performed on select prostate tissue specimens and coordinating prostaspheres. The TMPRSS-ERG fusion was found in approximately 60% of cancer cases tested. Surprisingly, the fusion was conspicuously absent from prostasphere cultures derived from TMPRSS-ERG+ tissues, even when the specimens contained >90% tumor. Analysis of monolayer cultures concomitantly derived from prostate tumor specimens also failed to demonstrate the presence of the gene fusion, indicating that both spheroid and adherent cultures select for fusion-negative cells[5].

Review of ETS Rearrangements in Cultured Prostate Epithelial Cells: The TMPRSS-ERG fusion has previously been identified in only one prostate cancer cell line, NCI-H660, derived from an androgen-independent small cell carcinoma of the prostate[7, 8]. None of the common prostate cancer cell lines including LnCaP, DU-145, PC-3, and CWR22 contain this fusion[6]. LnCaP and MDA-PCa2b were recently reported to contain rearrangement of the ETV1 gene to a prostate specific region resulting in aberrant expression with increased invasive activity[6]. The general inability to culture primary prostate cells that contain TMRPSS-ERG, and the under-representation of ETS rearrangements in prostate cancer cell lines is intriguing and suggests critical elements are absent in vitro, preventing the growth of these cells.

We have formulated three distinct possibilities why TMPRSS-ERG is not preserved in human prostate cells in vitro 1) prostate cancer stem cells responsible for propagating primary cells do not contain the TMPRSS-ERG fusion, rather it is a genetic event that occurs later in tumorigenesis as a result of genomic instability 2) Fusion-positive prostate cells undergo anoikis, apoptosis, or senescence unless additional growth/survival factors or stromal interaction is provided, or 3) genetically normal cells have a dramatic growth advantage over TMPRSS-ERG cancer cells, resulting in their rapid overgrowth.

Since the TMPRSS-ERG fusion is so prevalent in prostate cancer regardless of grade or stage, analyses of the genetic impact of these rearrangements is critical. Deciphering the fundamental survival factors necessary for culturing these cells will yield biological tools for the study of ETS rearrangements in addition to valuable insight into the vulnerabilities of these cells. Consequently, we proposed to define what factors are critical for survival and expansion of TMPRSS-ERG fusion-positive prostate cancer cells via the following aims:

Aim 1: Generate a collection of tumor specimens that contain the TMPRSS-ERG translocation, as demonstrated by FISH of the primary tumor.
   a. Generate xenografts from TMPRSS-ERG tissue specimens
   b. Generate prostasphere and monolayer (adherent) cultures from TMPRSS-ERG specimens in a variety of culture conditions, including altering media and additives (i.e., androgen, stroma)
   c. Generate stocks of cryopreserved dissociated prostate cells from TMPRSS-ERG specimens

Aim 2: Assess for the retention of the TMPRSS-ERG mutation in xenografts, expanded in vitro monolayer (adherent) cultures, and prostasphere cultures.

Aim 3: Assess the effect of inhibiting anoikis and/or apoptosis pathways in dissociated prostate epithelial cells derived from TMPRSS-ERG+ tissues on prostasphere formation via viral mediated gene transfer of genes that are known to disrupt these processes (i.e., Bcl-2, Ras, dominant negative p53).

III -KEY RESEARCH ACCOMPLISHMENTS:

The tasks of the training program include:

   1) Regularly meet with mentor to discuss career goals and progress
   2) Attend group meetings, journal clubs, and seminars related to research topics

3) Direct research project outlined in the proposal according to the specific aims:
   a. Develop a collection of TMPRSS-ERG fusion positive human prostate cancers
   b. Evaluate the ability to preserve the TMPRSS-ERG fusion in prostaspheres by varying culture conditions.
   c. Evaluate the ability to propagate TMPRSS-ERG+ cells in prostate epithelial monolayer cultures and in xenografts
   d. Perform viral mediated gene transfer of genes that block anoikis and apoptosis pathways in dissociated human prostate cells and evaluate the ability to maintain TMPRSS-ERG+ cells as prostaspheres.

Mentoring (Tasks 1 and 2): As I transition towards an independent research focus in human prostate stem cells and their role in tumor-initiation, my interactions with Dr. Witte continue in the form of meetings regarding ongoing and future collaborations and interactions with members of his research team to share data and discuss new strategies in prostate stem cell research.

Progress on Specific Aims:

Collecting TMPRSS-ERG+ samples: We have continued to expand our collection of human prostate tissue samples. We have collected approximately 70 additional benign and tumor specimens over the past year for experimental studies. We continue to work with the Tissue Procurement Core Laboratory (TPCL) at UCLA, with a pathology and technical staff that assist with tumor isolation from prostate specimens. After the prostate surgical specimens are removed en bloc, an experienced technician from TPCL prepares 5 or 6 prostatic sections ranging in thickness from 3-4mm. A sleeve of fresh tissue is obtained from the posterior (peripheral zone) of selected sections. Frozen slides are prepared and stained by H&E staining. An expert GU pathologist examines the slides and cancerous areas are marked and mapped to the remaining fresh tissue (see appendix figure 1). Tumor nodules were then dissected and isolated from benign tissue and processed via enzymatic and mechanical digestion into single cells.

Epcam/CD44/CD49f fractionation enables isolation of functionally distinct cells within the human prostate cellular hierarchy: Ongoing studies by our laboratory to delineate the human prostate cellular hierarchy have led to the discovery that the generally epithelial marker, Epcam, combined with basal markers CD44 and CD49f, enables isolation of prostate epithelial subpopulations with quiescent stem cells (SC), activated SC/progenitor (S/P) and terminally differentiated luminal cell (LC) functional characteristics. Epcam$^+$CD44$^+$ cells exhibit a basal expression profile, form spheres in vitro, and tubules in vivo (Figure 1). Although the majority of Epcam$^+$CD44$^-$ are incapable of sphere-formation and demonstrate a predominantly luminal cell profile, a subset of basal cells are present in this fraction, as evident by CD49f^{Hi} expression (Figure 1A and B). These Epcam$^+$CD44$^-$ cells induce robust tubule formation in vivo (Figure 1C). Since published data clearly demonstrates that CD49f^{Hi} expression is a requirement for tubule formation, additional fractionation of Epcam$^+$CD44$^{+/-}$ cells based on CD49f co-expression was performed. Approximately 60% of Epcam$^+$CD44$^+$ cells are CD49f^{Hi} and a 10-fold increase in sphere-forming activity is observed compared to Epcam$^+$CD44$^+$CD49f^{Lo} cells (Figure 1A and B). Approximately 30% of non-sphere-forming Epcam$^+$CD44$^-$ cells are CD49f^{Hi} and display nearly 20-fold higher tubule formation than Epcam$^+$CD44$^-$CD49f^{Lo} cells (Figure 1A and C). Therefore, Epcam$^+$CD44$^-$CD49f^{Hi} fractions likely represent a quiescent (non-sphere-forming) SC population that can be activated to form tubules in prostate tissue regeneration assays. On the other hand, Epcam$^+$CD44$^+$CD49f^{Hi} cells are proliferating S/Ps, capable of forming abundant spheres in vitro but relatively fewer tubules in vivo. This phenomenon is likely due to a loss of pluripotency as proliferating progenitors accumulate and outnumber *bona fide* SCs. Epcam$^+$CD44$^-$CD49f^{Lo} cells are clearly terminally differentiated LCs incapable of sphere-forming or tubule-inducing capability.

Epcam/CD44 fractionation enables enrichment of TMPRSS-ERG+ cells: In an effort to improve the retrieval of TMPRSS-ERG+ prostate cancer cells that are required for our studies, we utilized Epcam/CD44 fractionation to isolate luminal cells from tumors. In initial experiments with tumor tissues, however, it was noted that only a minor population of Epcam$^+$CD44$^-$ luminal cells remained

after standard 12-hour digestion with Collagenase (Figure 2A). This result was surprising, since the majority of cells present in tumor nodules should display Epcam$^+$CD44$^-$ (luminal) profiles. In order to evaluate whether or not standard enzymatic digestion procedures resulted in over digestion and loss of tumor cells, a series of experiments evaluating a variety of digestion times and collagenase concentrations was performed (data not shown). When digestion time was reduced to 4 hours in 0.25% collagenase, a marked shift in Epcam$^+$CD44$^-$ cells was noted, enabling optimization of luminal cells recovery (Figure 2B). FACS and RT-PCR of fractionated cells confirmed enrichment of basal Epcam$^+$CD44$^-$ and luminal Epcam$^+$CD44$^+$ fractions (Figure 2C and E). In order to confirm enrichment of tumor cells, PCR for TMPRSS-ERG fusion was performed on RNA isolated from cell fractions. Significant TMPRSS-ERG message was detected exclusively in the Epcam$^+$CD44$^-$ cell fraction (Figure 2E). This confirms that the luminal cell optimization procedure for enzymatic digestion of prostate tumor specimens enables robust recovery of tumor cells containing TMPRSS-ERG. This significant improvement in TMPRSS-ERG+ recovery may translate into an improved ability to expand primary prostate cancer cells in vitro and in vivo.

Recreating the tumor microenvironment to preserve tumor cells: In addition to enrichment of the TMPRSS-ERG+ cells, we have continued to focus on recreating the microenvironment that is conducive to prostate tumor growth. One factor that may enable cancer cell growth is the incorporation of support cells that recreate the tumor microenvironment. Although we have traditionally utilized rodent urogenital sinus mesenchyme to support human tissue regeneration in immunocompromised mice, it is possible that murine growth factors secreted by these cells may not be optimal stimulants of tumor growth. As a result, we have isolated stromal cells from fetal tissue, which demonstrate abundant growth potential and support benign human prostate tissue regeneration when combined with adult prostate cells or prostaspheres (see 2010 Progress Report). The use of fetal prostate stroma for tissue regeneration assays of primary prostate tumor cells has enabled retrieval of tumor grafts. Tumor nodules were dissected from high-grade surgical specimens and combined with human fetal prostate stroma and Matrigel prior to subcutaneous injection into NOD-SCID mice. Approximately 12-weeks following implantation, grafts were harvested and evaluated by immunohistochemistry (Figure 3). High-grade tumor foci were observed as well as areas of benign growth. Tumor foci demonstrated similar expression pattern of prostate markers as the original tumor (data not shown). The ability to regenerate primary tumors is a leap forward in our ability to identify and characterize human prostate stem cells. We are aggressively pursing tissue regeneration of more high-grade tumors with passaging in order to determine reproducibility of this technique. We also hope to begin to interrogate fractionated cells from tumors for their ability to function as cancer stem cells and trace the TMRPSS-ERG population throughout tumor development.

Niche Interactions of benign SC fraction may be exploited in tumors: The use of our human prostate tissue regeneration system and methods for separating prostate cells isolated from dissociated surgical specimens is a valuable tool for characterizing genetic events and cells of origin in prostate tumorigenesis. The ability to functionally distinguish cell populations that can interact with the niche to form benign or malignant-appearing tubular outgrowths is a huge step. It is clear, from our data, that Epcam$^+$CD44$^-$ tumor cells have acquired the capability to interact with the niche that is diminished in S/P cells and completely absent in benign LCs (Epcam$^+$CD44$^-$CD49f^{Lo}). The use of gene expression microarray analysis in future studies could enable pathways involved in this niche interaction to be identified and targeted as a future therapeutic strategy to prevent cancer progression/metastases in patients with advanced prostate cancer.

IV - REPORTABLE OUTCOMES:

We currently have four manuscripts in preparation that will be submitted within the next 3-6 months:

1) Guo, C, Zhang, B, and Garraway, IP. CD44 distinguishes quiescent human prostate stem cells from an activated sphere-forming stem/progenitor population. In preparation.

2) Zhang, B, Guo, C, An, D, and Garraway, IP. Human fetal prostate stroma supports benign and malignant prostate tissue regeneration with adult epithelial cells. In Preparation.

3)   Jing, J, Hindoyan, A, Goldstein, A, Lawson, D, Chen, D, Li, Y, Wang, S, Guo, C, Zhang, B, Gleave, M, Witte, O, Garraway, IP, and Wu, H. Identification of CD166 as a marker for enriching prostate tumor initiating cells. In preparation.

4) Lai, K, Lu, H, Zhang, B, and Garraway, IP. K13 is a marker of quiescent human prostate stem cells. In preparation.

Our data was presented at the Prostate Cancer Foundation Annual Retreat in Washington DC, September, 2010.

V - CONCLUSIONS:

Timeline for completion of research tasks documented in the original statement of work is listed below:

Months 0-6: Initiate cloning of viral vectors; obtain regulatory approval for human and animal research protocols.

Months 7-18: Collect prostate tissue specimens, attempt to establish new xenografts and monolayer cultures, and begin to evaluate TMPRSS-ERG fusion status in collected tissue specimens via FISH.

Months 19-30: Continue to collect tissue and evaluate for TMPRSS-ERG status. Begin altering growth conditions of dissociated cells that contain the translocation in attempt to preserve cells containing the fusion in vitro. Begin viral-mediated gene transfer of anti-anoikis and anti-apoptosis genes

Months 31-60:   Continue characterization of prostaspheres generated in altered growth environments and upon gene transfer of anti-anoikis/apoptosis genes. Evaluated ability to generate prostaspheres from newly established xenografts.

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APPENDIX:

FIGURE LEGENDS:

Figure 1: Differential functional capabilities of human prostate cell fractions. A. FACS of total prostate cells isolated from benign surgical specimens based on Epcam, CD44, and CD49f expression. Gates are set on predominantly basal (Epcam+CD44+) and predominantly luminal (Epcam+CD44-) fractions (middle panel) and then evaluated for CD49f expression (upper and lower dot plots). B. Sphere-forming assays are performed by culture of 10^4 fractionated cells in Matrigel. Unfractionated (U), Epcam+CD44+ (2) Epcam+CD44- (3) Epcam+CD44+CD49f^{Hi} (4) Epcam+CD44+CD49f^{Lo} (5). C. 12-week grafts generated from cell fractions. 1×10^5 fractionated cells were combined with 2×10^5 FPS and Matrigel followed by subQ implantation into NOD-SCID^{IL2grNULL} mice. Testosterone pellets were also implanted subQ to stimulate tubule formation.

Figure 2: TMPRSS-ERG fusion expression in fractionated prostate epithelial cells. The combination of Epcam and CD44 was used to isolate Epcam+CD44+ basal-enriched (A) and Epcam+CD44- luminal-enriched cell fractions (B) for quantitative RT-PCR. Fractionated cells were compared to total (unfractionated) cells. Relative increased expression of luminal markers (AR, PSA, CK8) in Epcam+CD44- fractions confirms enrichment in this population (D). Expression of TMPRRS-ERG is detected exclusively in the Epcam+CD44- fraction (E).

Figure 3: Prostate Cancer Regeneration. A slice of tissue from a radical prostatectomy specimen from a patient with high-grade (Gleason 5+4, pStage T3bN1M0) prostate cancer was procured with preparation of an adjacent frozen section. Tumor and benign tissue were separated and tissues were dissociated into single cells. 5×10^4 epithelial cells were combined with 1×10^6 fetal prostate stroma and injected subQ into NOD-SCID mice. After 5 months, grafts were harvested for histological analysis.

ATTACHED FIGURES:

    Figure 1
    Figure 2
    Figure 3
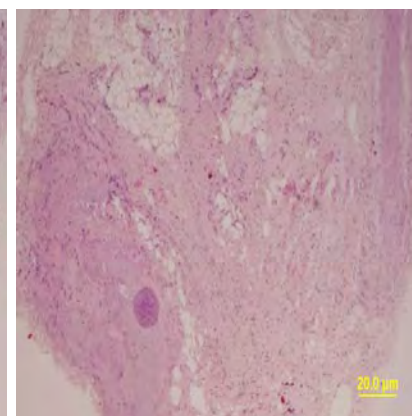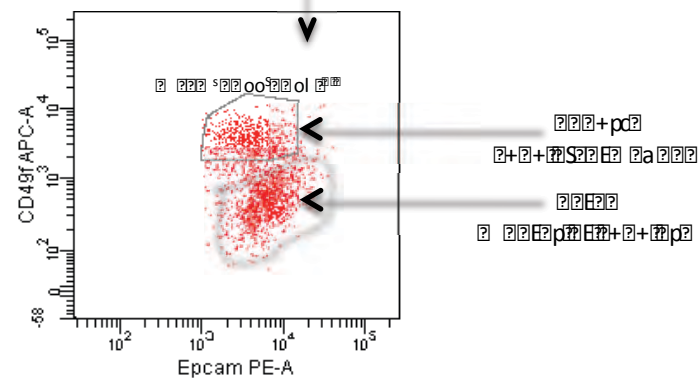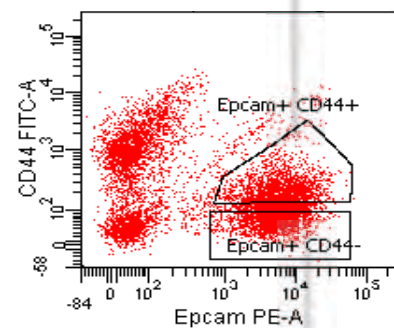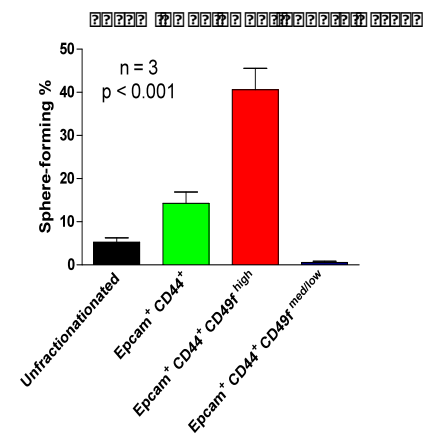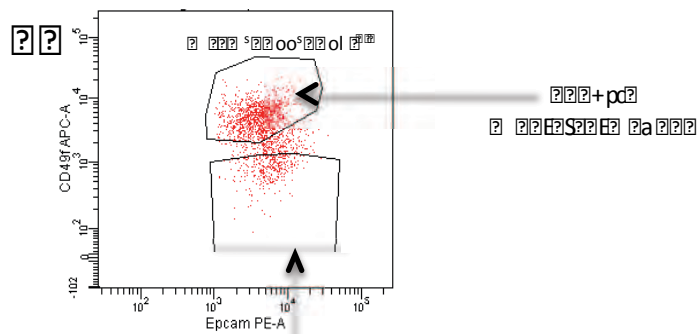
Epcam+ CD44+ CD49f APC-A

Epcam PE-A

CD44 FITC-A

Epcam+ CD44+

Epcam+ CD44-

Epcam PE-A

CD49f APC-A

Epcam PE-A

Epcam CD44 Sphere Assay

Sphere-Forming %

Unfractionated | Epcam+CD44+ | Epcam+CD44

$n = 3$
$p < 0.001$

Sphere-forming %

Unfractionationated | Epcam+ CD44+ | Epcam+ CD44+ CD49f high | Epcam+ CD44+ CD49f med/low

20.0 µm

CK8

CK14

P63

AR

PSA

CK5

TMPRSS2-ERG fusion gene

Epcam+ CD44+

Epcam+ CD44-

Epcam+CD44+

Epcam+CD44-

CD44 FITC-A

Epcam PE-A

Q1-2 Q2-2
Q3-2 Q4-2

Q1-3 Q2-3
Q3-3 Q4-3

Relative Gene Expression

Differential Expresion
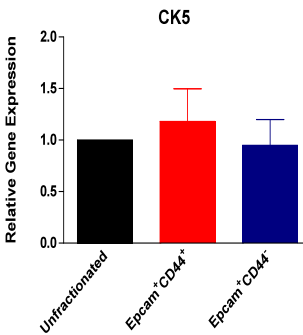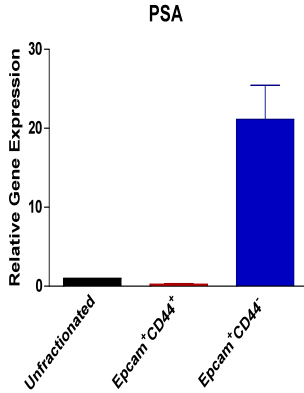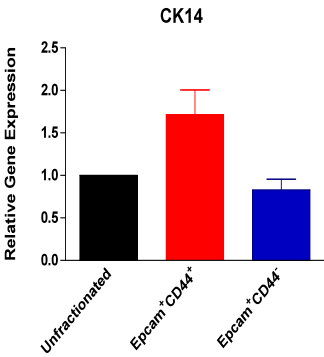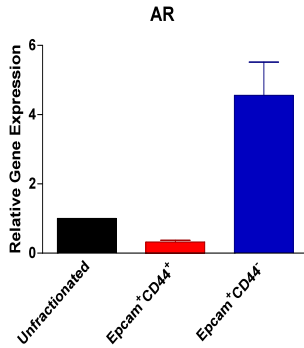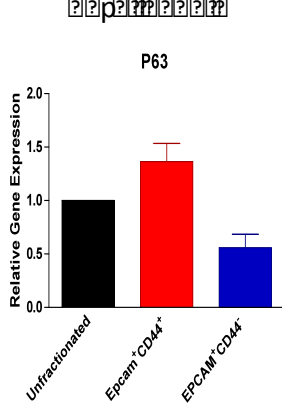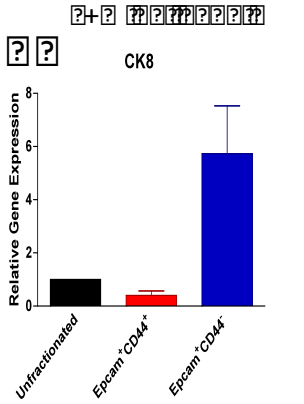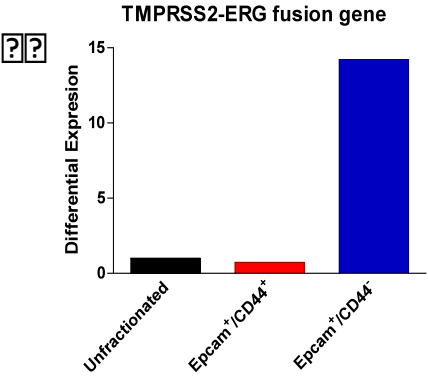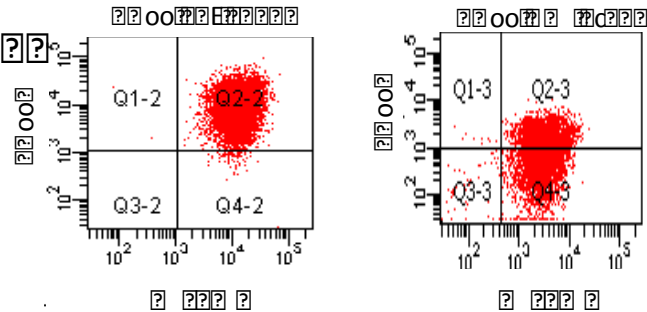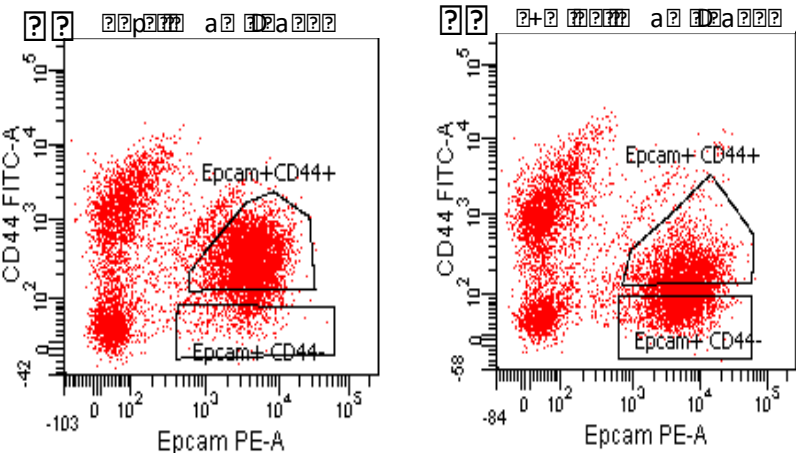
Unfractionated

Epcam⁺CD44⁺

Epcam⁺CD44⁻

Figure 3

Fetal Prostate Stroma Supports Primary Prostate Tumor Regeneration